(12) UK Patent Application (19) GB (11) 2 209 836 (13) A

(43) Date of A publication 24.05.1989

(21) Application No 8721789.9

(22) Date of filing 16.09.1987

(51) INT CL⁴
G01N 27/40

(52) UK CL (Edition J)
G1N NBGM N25B N25B3X N25DX
U1S S1517

(56) Documents cited
None

(58) Field of search
UK CL (Edition J) G1B BCB, G1N NBEX NBGM NBMX NBPM NBPX
INT CL⁴ G01N

(71) Applicant
Cambridge Life Sciences plc

(Incorporated in the United Kingdom)

Cambridge Science Park, Milton Road, Cambridge, CB4 4GN, United Kingdom

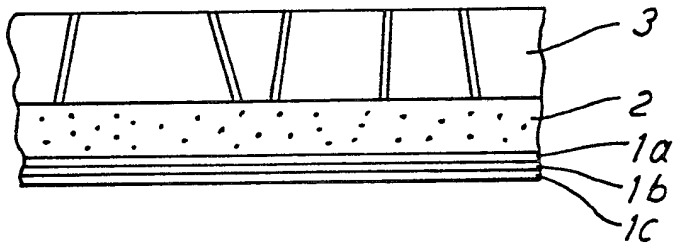
(72) Inventor
Peter Skelly

(74) Agent and/or Address for Service
D Young & Co
10 Staple Inn, London, WC1V 7RD, United Kingdom

(54) Multilayer enzyme electrode membrane and method of making same

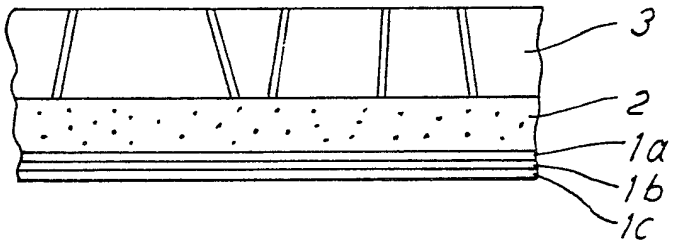
(57) A multilayer enzyme membrane for use in the polarographic detection of an enzyme substrate in a sample in the presence of a plurality of potentially interfering species. The membrane consists of a multilayer base (1a, 1b, 1c) supporting an immobilised enzyme-containing layer (2) and optionally covered by a microporous membrane (3). Each of the layers of the base are permeable to a polarographically active species produced by the reaction of the enzyme and its substrate, thereby to enable measurement of said species (and hence the substrate concentration) at an underlying electrode, but substantially impermeable each to a different one of the potentially interfering species, thereby to prevent that species from reaching the electrode. Since each layer of the base is thus specifically designed to eliminate a particular interfering species, multiple layer membranes can be built up having the capacity to eliminate any selected combination of interfering species according to need, instead of trying to design a single layer membrane capable of eliminating the interfering species all at the same time.

The layers are preferably formed by spin casting and have a thickness of 0.5-1.0 μm.



GB 2 209 836 A

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.


3
2
1a
1b
1c

MULTILAYER ENZYME ELECTRODE MEMBRANE AND METHOD OF MAKING SAME

This invention relates to a multilayer enzyme electrode membrane and a method of making same.

Polarographic cell systems employing an enzyme electrode membrane, i.e. a membrane having an enzyme immobilised therein or thereon, have been used extensively in recent years for the detection and measurement of various substances, particularly substances present in relatively small amounts in medical or clinical samples. A particular example is a glucose membrane comprising immobilised glucose oxidase and which may be used to measure blood sugar levels via the reaction:

$$\text{glucose} + O_2 \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconic acid} + H_2O_2$$

Although glucose itself is not polarographically active, both gluconic acid and hydrogen peroxide are, and are therefore detectable and measurable as a measure of the glucose content of the original sample. Other substances which can be detected and measured using similar techniques include uric acid, urea, cholesterol and some drug molecules.

Enzyme electrode membranes devised for this purpose generally comprise a sandwich-type construction i.e. comprising a layer of enzyme immobilised between two opposing porous films or layers, although some two-layer constructions have been proposed consisting simply of the porous backing layer or film and the immobilised enzyme. One of the first proposals in this field was for an enzyme electrode membrane comprising glucose oxidase immobilised between layers of cuprammonium regenerated cellulose (Cuprophane): Annals of the New York Academy of Science, Volume 102 pages 29-45 (1962). The Cuprophane layers, in that case, were permeable not only to the substance to be measured, i.e. glucose, but also to the products of the enzymic reaction, viz. gluconic acid and hydrogen peroxide, either of which could be detected, for example, by a pH electrode in the case of gluconic acid, or at a platinum anode in the case of hydrogen peroxide, see for example, US-A-3,539,455.

Since that time enzyme electrode membranes have become much more sophisticated, both in design and in the range of materials used, with

the object always of improving sensitivity and cutting out interference from other interfering species, i.e. species which interfere directly or indirectly with the final measurement, and which may be present either in the original sample, or produced as a by-product of the enzymic reaction.

The following is a representative selection of prior art disclosures:

GB-A-1,554,292. Yellow Springs Instrument Co. Inc. (Newman). This discloses an immobilized enzyme membrane for the polarographic determination of a hydrogen peroxide producing substance, e.g. glucose, comprising a first layer of silicone rubber, polymethyl methacrylate or cellulose acetate having a thickness of less than 2 microns and permeable to hydrogen peroxide but substantially impermeable to substances of higher molecular weight, a second layer, e.g. of porous polycarbonate, having a thickness of 1 to 20 microns, permeable to hydrogen peroxide, and the substance to be measured, i.e. glucose, but allegedly impermeable to other substances of higher molecular weight, and an ahdesive layer bonding the two, said adhesive layer consisting of or comprising the enzyme.

EP-A-0 079 502. Miles Laboratories Inc. (Oberhardt). This discloses a multilayer enzyme electrode membrane comprising a first relatively dense layer e.g. of cellulose acetate, on which are deposited an alternating array of porous polymer and immobilised enzyme layers, e.g. alternating layers of porous cellulose acetate deposited by a phase inversion technique, i.e. casting from a solution containing both a solvent and a non-solvent for the cellulose acetate, and layers of glutaraldehyde cross-linked glucose oxidase. The invention overcomes the problems allegedly associated with a single layer of cross-linked enzyme, by providing a more homogeneous distribution of enzyme throughout the membrane. Multiple layers of different porosity may be used.

EP-A-0 080 502. Miles Laboratories Inc (D'Orazio). This discloses an essentially two layer enzyme electrode consisting of a relatively dense layer, e.g. of cellulose acetate, and an overlying relatively porous layer, also of cellulose acetate, formed by phase inversion casting, and comprising the enzyme dispersed throughout the porous layer.

In both the Yellow Springs patent and the published applications by Miles, traditional casting techniques are used for both the base and subsequent layers, mainly solvent casting of the film onto a strippable substrate. Individual film thicknesses generally range from 1 to 20 microns,

with total membrane thicknesses in the range 40 to 100 microns, although suggestions are made in the Yellow Springs patent for sub-micron size support layers, i.e. the cellulose acetate, silicone rubber or polymethyl methacrylate layer, with suggested thicknesses in the range 0.5 to 1.0 micron, and an overall membrane thickness of less than 10 microns.

To be effective, enzyme electrode membranes for medical and clinical diagnostic purposes must possess a number of characteristics:

(1) they must be permeable to hydrogen peroxide or other molecular species to be measured;

(2) they must be impermeable to ascorbic acid, paracetamol and other molecular species which might otherwise interfere with the polarographic detection and measurememt of the hydrogen peroxide produced by the reaction between the enzyme and its substrate, i.e. the substance to be detected or determined;

(3) they should be impermeable to molecular species which interfere with the enzymatic reaction;

(4) they should give a rapid linear response;

(5) they should be strong enough to withstand cleaning;

(6) they should not delaminate on soaking;

(7) they should retain their enzymatic activity and response times over long periods.

Existing enzyme electrode membranes meet some but not necessarily all those requirements, and to a greater or lesser extent, leaving room still for the development of improved enzyme electrode membranes.

One of the particular problems in the design of new and improved enzyme electrode membranes is in the construction of the base layer of the membrane in view of the often conflicting requirements of physical strength, permeability to hydrogen peroxide, impermeability to other interfering species, bondability etc. etc., all of which are not necessarily found in one polymer. Relative impermeability to other interfering species is a particular problem, and is met in the present invention, not by using a single polymeric layer which possesses all the necessary requirements, but by using a laminated base structure composed of a plurality of thin films which collectively have the desired properties, and in particular the desired permeability to hydrogen peroxide and relative impermeability to other interfering species, such as ascorbic acid and paracetamol. In that way not

every layer need have the requisite combination of permeability and impermeability, so long as the overall combination does.

In accordance with the present invention, therefore, there is provided an enzyme electrode membrane for the quantitative determination of a given substance in a sample, said membrane comprising a polymeric base layer permeable to hydrogen peroxide, or other polarographically detectable product of the reaction between said substance and an enzyme reactive therewith, but substantially impermeable to at least one other molecular species present in said sample, or produced by said reaction, and being a species which might otherwise interfere with the polarographic detection of said product, and a layer consisting of or comprising an immobilised enzyme reactive with said substance bonded to the base layer, wherein said base layer consists of a plurality of sub-layers bonded one to the other in superimposed relation, each of said sub-layers being permeable to said product, but being of different permeability with respect to at least one of said competing species, as compared with the other sub-layer or at least one of the other sub-layers, with the proviso that at least one of said sub-layers is substantially impermeable to at least one of said competing species.

By using sub-layers each of a different permeability with respect to a given interfering species, but with a common permeability to the desired polarographically detectable reaction product, enzyme electrode membranes can be tailor made to meet different requirements. For example, enzyme electrode membranes for blood sugar determination can now be tailor made with either paracetamol rejection or ascorbic acid rejection, or both, by specifically incorporating into the base a sub-layer impermeable to paracetamol, and/or a sub-layer impermeable to ascorbic acid, but permeable nevertheless to hydrogen peroxide, and without necessarily having all the other characteristics which would be necessary were that particular material alone to be used as the base of the enzyme membrane.

Preferably, the multi-layer base of the enzyme electrode membrane of this invention is composed of a plurality of sub-layers each of sub-micron thickness, i.e. less than 1 micron thick, preferably in the range 0.5 to 1 μm, although thicknesses can go as low as 0.1 μm, with total thicknesses, of the composite base layer, up to 5 μm. Such ultra-thin layers can be obtained by the technique of spin casting in which a solution of the polymer is deposited on a flat plate having a surface, e.g. of polyethylene terephthalate

(MELINEX), polypropylene (PROPAFILM) or siliconised glass from which the film can be subsequently stripped, and spinning the plate to spread the solution over the surface of the plate as an ultra-thin film, and from which the solvent is then removed, e.g. by evaporation, thereby to deposit the polymer as an ultra-thin film on said plate.

One of the specific benefits of using spin casting in the production of the thin films used to form the base of the enzyme electrode membrane of this invention is that, because the individual films are so thin, polymeric materials can be used which could not previously be used owing to the low permeability of thicker films to hydrogen peroxide, but which in ultra-thin form do have sufficient hydrogen peroxide permeability to be operable.

In another aspect, therefore, the present invention provides a method of producing an enzyme electrode membrane which comprises forming a plurality of spun cast polymeric films each having a thickness of 1 micron or less, superimposing said plurality of films one on top of the other to form a polymeric base for the enzyme electrode membrane, said polymeric base being permeable to hydrogen peroxide or other polarographically detectable molecular species formed by the reaction of the enzyme and the substance to be determined, forming on said base a surface layer consisting of or comprising the immobilised enzyme, and optionally bonding to the surface of the immobilised enzyme containing layer a further polymeric layer, said further layer at least being permeable to the substance to be determined.

In performing the above method, the steps can be performed in any order. For example, and most preferably, the base of the membrane is first formed by spin casting the individual films directly one on top of the other. Once the base has been formed the immobilised enzyme containing layer is formed in situ on the base in known manner, for example, by applying to the surface of the base a paste comprising the enzyme in admixture with a cross-linking agent such as glutaraldehyde or hexamethylene diisocyanate, thereby to cross-link the enzyme in situ on the surface of the base, or by applying to the base a gelatin solution to which the enzyme has been added, or by the technique disclosed in EP-A-0 080 502, i.e. phase inversion casting of a solution containing the enzyme in admixture with a suitable polymeric matrix matrial, e.g. cellulose acetate. As the final step, the immobilised enzyme layer may or may not be protected by the optional upper, e.g. microporous, surface film or layer.

Alternatively, the membrane may be made in reverse order, i.e. by first forming an immobilised enzyme layer, with or without the microporous protective layer, and spin casting the base layers directly onto the immobilised enzyme layer.

A section through a typical membrane construction according to the invention, and prepared by the above method is shown in the accompanying drawing. According to the invention, the membrane is composed of a plurality of ultra-thin, preferably spun cast, base layers 1a, 1b, 1c, an immobilised enzyme layer 2, and optionally the microporous surface layer 3.

As already indicated, the technique of this invention enables a wide range of polymeric materials to be selected for the base layers of the membrane, including polymeric materials not previously considered suitable because of their relatively low permeability to hydrogen peroxide. Included in this category is, for example, cellulose propionate, which can now be incorporated into the base as an ultra-thin sub-layer to provide substantial impermeability to paracetamol. Similarly polyelectrolyte films, e.g. of polyacrylic acid, can be incorporated to provide impermeability to ascorbic acid without substantially reducing the permeability to hydrogen peroxide and thus without substantially affecting the response time or sensitivity of the membrane. Indeed, in many cases the use of ultra-thin spun cast films as the base of the membrane gives substantially reduced, i.e. quicker, response times, and increased sensitivity.

Included in the range of polymeric materials which can be used to form one or more of the sub-layers of the base to the enzyme membrane of this invention are:

<u>Cellulose Acetate Esters</u>:

Cellulose acetate
Cellulose acetate butyrate
Cellulose acetate methacrylate
Cellulose acetate phthalate
Cellulose acetate propionate
Cellulose acetate valerate

<u>Cellulose Esters</u>:

Cellulose triacetate
Cellulose tributyrate
Cellulose isobutyrate

Cellulose methacrylate
Cellulose triphthalate
Cellulose tripropionate
Cellulose trivalerate

Cellulose Ethers:

Ethyl cellulose
2-cyanoethyl cellulose

Non-Cellulosics:

Polyvinyl acetate
Polyvinyl alcohol (non-water soluble grades)
Ethylene-vinyl acetate copolymers
Polyvinyl butyral
Polyvinyl formal
Polyamides, e.g. nylon 6, nylon 4,6, nylon 6,6, and substituted polyamides e.g. methoxynylon
Polyvinyl cinnamate
Polyvinyl stearate
Polyhydroxy ethyl methacrylate
Polyhydroxy propyl methacrylate
Polyhydroxy butyl methacrylate
Polyhydroxy propyl acrylate
Polyethylene vinyl alcohol copolymers
Polyethylene imine

and many other commercially available homo-, co- and ter-polymers.

Alternatively, water soluble polymers can be used which are then subsequently cross-linked by thermal, chemical, ultraviolet light or high energy radiation means, these techniques being well known to those skilled in the art. Polymers of this nature which can be used include:-

Polyvinyl alcohol (water soluble grades)
Polyvinyl pyrollidone
Polyacrylic acid or its salts
Polymethacrylic acid or its salts
Polystyrene sulphonate
Sodium carboxymethyl cellulose
Sodium carboxymethyl 2-hydroxyethyl cellulose
2-hydroxy ethyl cellulose

Methyl cellulose

2-hydroxy ethyl methyl cellulose

2-hydroxy butyl methyl cellulose

2-hydroxy ethyl ethyl cellulose

2-hydroxy propyl cellulose

Ethyl 2-hydroxy ethyl cellulose

Combinations or blends of two or more of the afore-mentioned polymers can be used together, by being dissolved in a co-solvent and spun together into the substrate. In this way a base layer or layers with two or more functional groups present can be made which can be advantageous to the properties of the base layer.

As the immobilised enzyme there may be used any enzyme capable of reacting with the substance (enzyme substrate) to be determined to produce a polarographically detectable species, e.g. hydrogen peroxide. Typical examples are glucose oxidase for the determination of glucose, cholesterol oxidase for the determination of cholesterol and urease for the determination of urea, and uric acid. The enzyme may be immobilised in the membrane by various techniques already mentioned.

Finally the immobilised enzyme layer may be further protected by outer or upper layers, e.g. a microporous or ultra-filtration membrane, permeable at least by the substance to be determined, and preferably substantially impermeable to larger molecular species which may be present in the sample and which may otherwise interfere with the enzymatic reation and/or the subsequent polarographic measurement. Such protective membranes and techniques are known and need not be further described. The preferred microporous or ultra-filtration membrane material used to protect the enzyme layer in the membrane of this invention is a microporous polycarbonate, e.g. the material sold under the trade name NUCLEPORE.

Membranes according to this invention, and the method of producing them, are illustrated by the following examples.

EXAMPLE 1

Glucose Biosensor Membrane with Paracetamol Rejection

A membrane suitable for a glucose biosensor showing low interference to the drug paracetamol, (present in the blood of persons taking analgesics) is manufactured in the following way.

A polymer solution of 5% methoxy nylon, ELVAMIDE 3061, is made by dissolving 5 grams of the polymer in 47.5 grams of methanol "ANALAR" and 47.5 grans of chloroform "ANALAR". When the dissolution is complete, the solution is ready for use.

Additionally, a solution of cellulose propionate is also made by dissolving 10 grams of cellulose propionate in 90 grams of acetone.

Thirdly an enzyme solution is made by dissolving 0.1 grams of glucose oxidase in 8 mls of phosphate buffer (pH 7) and when the enzyme has dissolved completely a 2 ml solution of glutaraldehyde (25% aqueous) is injected into the solution and quickly stirred in.

A piece of polyethylene terephthalate sheet, MELINEX, is placed on the vacuum chuck of a vacuum photoresist spinner and cleaned by spinning 5 mls of filtered acetone over the surface; during this procedure the spinning speed is adjusted to 1400 rpm. A small amount of the 5% ELVAMIDE solution, 5 ml, is then placed in the centre of the MELINEX sheet and spun at 1400 rpm for 1 minute.

Next 5 ml of the cellulose propionate solution is placed on the spun film of methoxynylon, still on the chuck, and the chuck respun at 8000 rpm for 1 minute. As a result a thin film of cellulose propionate is spun on top of the methoxynylon film.

1 ml of the freshly prepared enzyme solution is placed on the centre of the spun cellulose propionate film and spun for 5 seconds at 1400 rpm.

Then a disc of polycarbonate Nuclepore membrane (0.03 µm pore size) is brought into contact with the enzyme layer surface by being applied on a small hand roller. The membrane is found to stick well to the surface. It is then removed intact from the vacuum chuck and placed between glass plates under pressure after having a MELINEX sheet placed on top. The assemblage is placed in an oven at 45°C for 30 minutes to ensure chemical reaction and adhesion.

When the heating period is complete, the assembly is removed from the oven, allowed to cool and then separated from the surfaces of the glass.

The membrane is then peeled off the surface of the MELINEX and the O-rings attached adhesively to the base side. The individual O-rings are then cut into individual pieces and packed in dry cool conditions. Such a membrane is suitable for use in an electrochemical sensor and can be used to measure blood glucose concentration in the physiological range, when used with a calibration solution.

It is found that the membrane shows linear correlation with blood glucose concentrations, but negligible response to normal physiological levels of paracetamol. The membrane is therefore very suitable for measuring blood sugar levels of patients who hve taken paracetamol tablets before measurement.

EXAMPLE 2

Glucose Membrane with Ascorbate Rejection

A membrane suitable for measuring blood glucose using a glucose biosensor can be manufactured in the following way. Such a membrane has the special characteristic that ascorbate ions present due to the injection of vitamin C tablets are not able to penetrate the membrane and give false readings at the biosensor eiectrode surface.

The means of manufacture of the membrane is similar to that of Example 1, except that in place of the cellulose propionate solution there is used a solution of 5% polyacrylic acid in water neutralised to pH 7 with sodium hydroxide solution which is spun at 5000 rpm on top of the methoxynylon layer.

Due to the exclusion effect, DONNAN, such a membrane gives a linear response to blood glucose concentrations but is not affected by high concentrations of the ascorbate ion found in patients taking large amounts of vitamin C tablets.

CLAIMS

1. An enzyme electrode membrane for the quantitative determination of a given substance in a sample, said membrane comprising a polymeric base layer permeable to hydrogen peroxide, or other polarographically detectable product of the reaction between said substance and an enzyme reactive therewith, but substantially impermeable to at least one other molecular species present in said sample, or produced by said reaction, and being a species which might otherwise interfere with the polarographic detection of said product, and a layer consisting of or comprising an immobilised enzyme reactive with said substance bonded to the base layer, wherein said base layer consists of a plurality of sub-layers bonded one to the other in superimposed relation, each of said sub-layers being permeable to said product, but being of different permeability with respect to at least one of said competing species, as compared with the other sub-layer or at least one of the other sub-layers, with the proviso that at least one of said sub-layers is substantially impermeable to at least one of said competing species.

2. An enzyme electrode membrane according to claim 1, wherein the sub-layers of the base have individual thicknesses in the range 0.5 to 1 micron.

3. An enzyme electrode membrane according to claim 2, wherein said sub-layers are formed by spin casting.

4. An enzyme electrode membrane according to claim 1, 2 or 3, wherein the base comprises one or more layers of methoxynylon.

5. An enzyme electrode membrane according to any one of claims 1-4, wherein the base comprises one or more layers of cellulose propionate.

6. An enzyme electrode membrane according to any one of claims 1-5, wherein the base comprises one or more layers of polyacrylic acid.

7. An enzyme electrode membrane according to any one of claims 1-4, wherein the immobilised enzyme is glucose or cholesterol oxidase.

8. An enzyme electrode membrane for a glucose biosensor, comprising a layer consisting of or comprising immobilised glucose oxidase supported on a hydrogen peroxide permeable polymeric base, wherein the base comprises a plurality of spun cast polymeric layers each having a thickness in the range 0.5 to 1 micron, at least one of said layers consisting of spun cast methoxynylon, and at least one other of said layers consisting of spun cast cellulose propionate or spun cast polyacrylic acid.

9. An enzyme electrode membrane according to claim 8, wherein said base consists of a first layer of spun cast methoxynylon and a second layer of spun cast cellulose propionate or spun cast polyacrylic acid.

10. An enzyme electrode membrane according to any one of the preceding claims, wherein the exposed surface of the immobilised enzyme is protected by an overlying layer of polymeric material which is permeable to the substance to be determined.

11. An enzyme electrode membrane according to claim 10, wherein said protective layer is composed of a microporous polycarbonate.

12. A method of producing an enzyme electrode membrane which comprises forming a plurality of spun cast polymeric films each having a thickness of 1 micron or less, superimposing said plurality of films one on top of the other to form a polymeric base for the enzyme electrode membrane, said polymeric base being permeable to hydrogen peroxide or other polarographically detectable molecular species formed by the reaction of the enzyme and the substance to be determined, forming on said base a surface layer consisting of or comprising the immobilised enzyme, and optionally bonding to the surface of the immobilised enzyme containing layer a further polymeric layer, said further layer at least being permeable to the substance to be determined.

13. A method according to claim 12, wherein said sub-layers of the base are spun cast to a thickness in the range 0.5 to 1 micron.

14. A method according to claim 12 or 13, as applied to the production of a membrane as claimed in any one of claims 1-11.

15. A method according to claim 12, 13, or 14, which comprises successively spin casting one on top of the other the layers of polymeric material which make up the base, applying to the spun cast, multi-layer base an immobilised enzyme layer, and optionally applying thereover the further protective layer of microporous polymeric material.

16. A method according to claim 12, 13 or 14, which comprises forming a layer consisting of or comprising an immobilised enzyme optionally superimposed on a layer of microporous polymeric material, and successively spin casting onto the enzyme layer the polymeric layers which make up the base of the membrane.

Published 1988 at The Patent Office, State House, 66 71 High Holborn, London WC1R 4TP. Further copies may be obtained from The Patent Office, Sales Branch, St Mary Cray, Orpington, Kent BR5 3RD. Printed by Multiplex techniques ltd, St Mary Cray, Kent. Con. 1 87